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| (54) Title: ULTRAHIGH RESOLUTION COMPARATIVE NUCLEIC ACID HYBRIDIZATION TO COMBED DNA FIBERS | | |
| (57) Abstract A method for detecting and quantifying the presence of a nucleic acids sequence in a genome comprising: (A) immobilizing by combing the target nucleic acids sequence on a supportive matrix, (B) contacting the combed nucleic acids sequence with a 1:1 mixture of differently labeled nucleic acids probe to be tested and reference nucleic acids probe to form hybridization, (C) counting individual labeled hybridization dots located on combed nucleic acids sequence, (D) determining the number of the nucleic acids sequence to be detected and quantified by the calculation of the ratio between the total dot number from the test probe and the total dot number from the reference probe. | | |

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ULTRAHIGH RESOLUTION COMPARATIVE NUCLEIC
ACID HYBRIDIZATION TO COMBED DNA FIBERS

BACKGROUND OF THE INVENTION

5 This invention relates to the detection and quantification of the presence of a gene in a genome. In one embodiment, the invention relates to the detection and quantification of a human oncogene.

10 A recently developed method, called comparative genomic hybridization (CGH) (Kallioniemi et al. 1992, du Manoir et al. 1993; Joos et al. 1993) has provided a new tool to detect non-random gains and losses of DNA sequences in genomic DNA (obtained e.g., from tumor specimens). For CGH, genomic tumor DNA is labeled with a hapten (e.g., Biotin) or directly with a
15 fluorochrome (e.g., FITC). Genomic DNA prepared from normal cells (of the patient or other persons) is differently labeled with another hapten (e.g., digoxigenin) or directly with another fluorochrome (e.g., TRITC or Texas red). Labeled tumor and control DNAs are mixed in equal amounts. This mixture is
20 hybridized in the presence of an excess of unlabeled cot1-DNA to normal metaphase spreads (target chromosomes) prepared from a healthy male or female person. The excess of cot1-DNA hybridizes to labeled interspersed and tandem repetitive sequences present in genomic DNA as well as to such sequences
25 present in the target chromosomes. This step is essential to suppress the unwanted hybridization of the repetitive sequences to the target chromosomes (Lichter et al. 1988, Pinkel et al. 1988). Following hybridization and washing steps, standard detection procedures are applied to visualize haptenized
30 sequences with two different fluorochromes (Lichter and Cremer 1992). This step is omitted in case of DNA-probes directly labeled with fluorochromes.

35 Consider for example a tumor with an essentially diploid karyotype except for a few monosomic or trisomic chromosomes or chromosome segments. Labeled DNA fragments with a size of

several hundred base pairs from the tumor DNA and the normal control DNA will hybridize with equal probability to their respective target sequences. The labeled DNA fragments from a chromosome or chromosome segment present in two copies in the pseudodiploid tumor cells together with the differently labeled fragments from genomic DNA of diploid cells yield a certain color mixture on the respective target chromosome or chromosome segment, e.g., yellow when the chromosome or chromosome segment is labeled with equal numbers of green and red fluorochromes representing the hybridized fragments from tumor DNA and normal DNA, respectively. For a chromosome segment present in three or higher copy numbers in the tumor, this color would become more greenish, while the loss of the segment would result in a more red color.

These color changes can be quantitatively recorded by measuring fluorescence ratio profiles along target chromosomes (du Manoir 1994, Piper et al. 1994). The choice of the appropriate equipment to measure signal intensities is important. Detectors should allow linear intensity measurements over a wide range. CCD-cameras are particularly useful in this respect. All data are stored digitally so that they can be used by microprocessor for the calculation of fluorescence ratios. In this way, a copy number karyotype can be established.

CGH can be performed with DNA extracted from archived, paraffine embedded tissues. Even minute amounts of genomic DNA can be used for this purpose after amplification with degenerate oligonucleotide primers (DOP-PCR) (Telenius et al. 1992; Speicher et al. 1993; Isola et al. 1994). It is possible to microdissect areas containing tumor cells from a tissue section and screen it for gains and losses of genetic materials by CGH performed with DOP-PCR amplified DNA (Speicher et al. 1994).

The number of studies, which demonstrate the usefulness of CGH to detect DNA copy number changes in tumors, is rapidly

increasing. Studies published so far reflect already a variety of tumors, including various forms of acute and chronic leukemias, bladder cancer, breast cancer, colorectal cancer, gliomablastoma, kidney cancer, neuroblastomas, prostate cancer, small cell and non-small cell lung carcinomas, uvea melanomas (e.g., du Manoir et al. 1993, 1994; Isola et al. 1994a,b; Joos et al. 1993; Kallioniemi et al. 1992, 1993, 1994a,b; Muleris et al. 1994; Ried et al. 1994; Schrock et al. 1994, Speicher et al. 1993, 1994, 1995 and our unpublished data). Numerous hitherto unknown regions, in particular amplification sites, have been found in these studies and will become the focus of efforts to clone the respective tumor relevant genes. Different tumor entities generally show distinctly different patterns of non-random changes and clinical follow up studies will show to which extent specific gains and losses can be correlated with the clinical course and prognosis of a given tumor.

The minimum size of a chromosome segment for which a single copy number change can be detected at present by CGH is in the order of 10 Mbp (Joos et al. 1993; du Manoir et al. 1994; Piper et al. 1994). Possibly, the resolution can be somewhat improved, when CGH is performed on prometaphase chromosomes. For amplified DNA sequences the detection limit of CGH is presently about 2 Mbp (number of amplification repeats times amplicon size). Still the precision with which the borders of chromosome segments involved in gains or losses is limited by the banding resolution of the target chromosomes. Thus, the current status of the CGH development does not allow to define the copy number representation of single tumor relevant genes (e.g., oncogenes, tumor suppressor genes). This limits the application of CGH to the detection of gross, unbalanced chromosomal abnormalities.

SUMMARY OF THE INVENTION

This invention aids in fulfilling these needs in the art by providing a method of ultrahigh comparative genomic

hybridization, which differs from state-of-the-art comparative genomic hybridization by the following new and essential features. The approach is performed on combed DNA fibers instead of reference chromosomes and referred to as combed
5 fiber CGH. Combed fiber CGH allows the analysis of copy number representation of specific sequences (represented by the combed DNA fibers) in a genomic test DNA with an ultrahigh resolution (in the kb-pair range instead of the Mb-pair range as previously published methods). This improvement makes combed
10 fiber CGH a very useful method to study the copy number representation of single genes or parts thereof.

Combed fiber CGH is particularly suited to eliminate background problems in fluorescence measurements, which arise when the fluorescence is measured from entire DNA-spots. In
15 the case of combed fiber CGH, the area of fluorescence measurements is adapted to a single fiber in a way that only hybridization dots located precisely on the DNA fiber contribute to the measured signal derived from the test or reference genomic DNAs. This improvement provides a very
20 considerable advantage of combed fiber CGH as compared to a CGH approach where fluorescence measurements are obtained from entire DNA-spots of non-ordered target DNA sequences attached to a supportive matrix.

In addition to fluorescence intensity measurements, which
25 can be carried out on individual combed target DNA fibers by standard procedures, combed fiber CGH allows the counting of hybridization dots located on the combed DNA fibers. By this approach the total number of dots on a sufficiently large series of target DNA fibers resulting from hybridized test and
30 a reference genomic DNA can be counted and a ratio (or a difference) between the total dot number from the test genomic DNA and the total dot number from the reference genomic DNA can be calculated as a measure of the copy number representation of the combed target DNA sequence in the test and reference
35 genomic DNA. This approach has not been realized so far in CGH

experiments and allows the evaluation of combed fiber CGH experiments in situations where the resulting signals are too weak to allow meaningful measurements of fluorescence ratios on single DNA fibers.

5 Instead of 1:1 mixtures of test and reference genomic DNAs, 1:1 mixtures of RNA preparations (or from cDNAs synthesized from RNA preparations) representing equivalent numbers of test and reference cells can be hybridized under suppression conditions (see standard CGH procedures) to combed
10 target DNA fibers e.g., cDNA fibers representing the coding sequence of genes of interest. The evaluation of the experiment is performed as described above. This approach allows an estimate of the relative copy number representation of mRNAs in test cells (e.g., tumor cells) and reference cells
15 (e.g., normal progenitor cells of the tumor cells).

 Target DNA fibers, e.g., cosmids containing (part of) a gene of interest are genetically engineered in a way that interspersed repetitive sequences are removed to avoid problems of insufficient suppression hybridization. (Note: only target
20 fiber specific single copy sequences, but not interspersed repetitive sequences contained in the hybridization mixture, can hybridize to the combed target DNA fibers under these precautions.)

 In case the localization of individual target DNA fibers
25 cannot be easily identified by arrays of numerous signal dots along each fiber, several procedures can be followed for an unequivocal target fiber identification with fluorochromes that have an emission spectrum, which allows a clear distinction from the emission spectrum of the fluorochromes implied in the
30 visualization of hybridized test and reference genomic DNA fragments. a) Fibers can be stained with appropriate fluorescent DNA stains. b) Target fiber DNA can be cloned in the presence of fluorochrome labeled DNA nucleotides for the direct visualization of the fiber or in the presence of hapten
35 modified nucleotides, e.g., BRdU, biotin, digoxigenin, for

indirect visualization by, e.g., indirect immunofluorescence. c) Target fiber DNA can be visualized by the addition of an appropriate amount of labeled target DNA sequences to the hybridization mixture employed for combed fiber CGH. In this case, the amount of labeled target sequences should not be excessive in a way that suppresses the hybridization of target specific labeled test and genomic DNA sequences. d) Target fiber DNA containing vector sequences can be hybridized with labeled vector sequences added to the hybridization mixture. If desirable, linker DNA of different length can be added to target fiber DNA in a way that makes it possible to identify the course and orientation of the combed target fiber by signals derived from the addition of labeled linker DNA sequences to the hybridization mixture.

Instead of fluorochromes with different emission spectra, fluorochromes that differ in fluorescence lifetime can be used. The choice of fluorochromes is performed with particular reference to avoid background fluorescence from the supportive matrix as much as possible.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENT

This invention involves the use of a DNA alignment method for the detection and quantification of multiple copies of a gene present in a genome. By multiple copies is meant visualization of at least about 100 copies per genome, with very good results at about 2000 copies per genome.

Ultrahigh Resolution Comparative

Nucleic Acid Hybridization To Combed DNA Fibers

In the following part we describe the development of a CGH-test providing a resolution at the single gene level. This test can be fully automated and broadly used in clinical settings.

The new test is based on the idea that, instead of entire chromosomes, specific target nucleic acids (DNAs or RNAs) are immobilized on a supportive matrix, such as glass or plastic

materials, in any desirable geometric format. The number of target nucleic acid (TNA-) spots and the sequence complexity of each TNA-spot can be chosen with regard to the specific goals of a test (see the application examples below). The number of TNA-spots included in a given matrix-CGH test may vary from a few spots to hundreds or even thousands of spots (for potential applications see below).

A typical TNA-spot may contain DNA from a single cosmid representing a gene or part of a gene of interest or it may contain a complex mixture of DNA representing a chromosome segment or even an entire chromosome of interest. In the latter case a matrix CGH test would not provide a resolution superior to the resolution of CGH to reference metaphase chromosomes. In the following we will construct our considerations mainly to the development of a matrix CGH test with the highest conceivable resolution, i.e., a test to detect copy number changes in a set of selected genes.

A matrix with TNA-spots as described above can be used to test tumor or other test DNAs for genetic imbalances down to the kbp-range. For this purpose, the hybridization probe consisting of a 1:1 mixture of differently labeled test and reference genomic DNAs (or RNA- or cDNA-preparations) is hybridized under suppression conditions against the set of immobilized TNA-spots. Measurements of the fluorescence ratio on each individual TNA-spot should provide an estimate of the copy number representation of the respective target sequences in the test DNA (or test RNA) as compared to the reference DNA (or reference RNA) (for further details of measurements see below).

The successful development of such a test depends on three requirements, namely the ability to firmly immobilize target nucleic acids on a supportive matrix, e.g., glass or plastic, a low autofluorescence of the matrix, and a sufficiently high signal/noise ratio for sequences specifically hybridized to a given TNA-spot. Notably, in CGH experiments with two

differently labeled genomic DNAs the fraction of labeled DNA fragments, which are specific for a given TNA-spot, is generally very small. Any non-specific attachment of labeled sequences or detection reagents to the matrix may impair or even inhibit the measurement of meaningful fluorescence ratios. Such adverse effects may become a limiting factor in attempts to measure fluorescence ratios on entire TNA-spots, particularly in cases where the specific signal is relatively small as compared to background.

In order to minimize background problems in fluorescence ratio measurements we make use of a procedure called "molecular combing" (Bensimon et al. 1994), which is relied upon and incorporated in its entirety by reference herein. By this procedure DNA target fibers can be extended and aligned in parallel like hairs by the use of a comb. To this end, the DNA fibers are attached at one end to a solid surface, "combed" by a receding air-water interface, and finally immobilized on the drying surface. (Bensimon et al. 1994). In this way the DNA of each TNA-spot can be represented by a series of "combed" target DNA fibers, all representing a specific DNA sequence of interest, e.g., a cosmid clone from a gene of interest. Using standard fluorescence in situ suppression hybridization techniques under appropriate stringency conditions, complementary sequences present in the hybridization probe hybridize specifically to these target sequences.

For each TNA-spot, fluorescence is separately recorded for both fluorochromes on a series of individual combed target DNA fibers using an appropriate camera, such as a CCD-camera. For evaluation, each target sequence is enclosed in a narrowly adapted rectangular field to determine the fluorescence of the fluorochromes applied in the labeling/visualization of the hybridized DNA fragments. Background fluorescence is measured in the immediate neighborhood of each measured target sequence and subtracted. After background subtraction, the fluorescence ratio is determined for each individual target

sequence with a microprocessor. For each TNA-spot the variation of fluorescence ratios obtained for a number of combed target DNA fibers is determined. Target sequences, which are represented in normal copy numbers in both the test and the reference genomic DNA, are used to obtain reliable thresholds for ratios indicative for increased or decreased copy number representation. For TNA-spots representing target sequences, which are over- or underrepresented in the test-DNA, one should then obtain correspondingly increased or decreased fluorescence ratios, while balanced regions should yield a ratio within the limits of control experiments. Since fluorescence ratios are recorded from individual target DNA fibers present in the TNA-spots, the new test is inert against variations in the total amount of target-DNA in each TNA-spot.

In model experiments, probe mixtures consisting of different ratios of biotin labeled and digoxigenin labeled cosmid sequences (e.g., 1:1 (20 ng + 20 ng), 2:1 (20 ng + 10 ng), 5:1 (20 ng + 4 ng) and 10:1 (20 ng + 2 ng)) were prepared. For each chosen ratio of sequences in the probe mixture, the same cosmid was used as target sequence. Following comparative hybridization some fifty target DNA fibers were evaluated as described in the Examples. The results demonstrate highly significant differences of mean fluorescence ratio values obtained for the different probe mixtures.

The use of individual target DNA fibers provides the possibility of another approach for the evaluation of a CGH experiment. Instead of measuring fluorescence intensities from entire target DNA fibers, it is possible to simply count individual fluorescence hybridization dots. Each such dot presumably represents the hybridization of an individual probe DNA fragment of a few hundred bp. The coverage of the combed target DNA sequence with differently colored dots from the 1:1 hybridization mixture of genomic DNAs is a stochastic event. The dot numbers for the two differently labeled genomic DNAs counted over a series of target DNA DNA fibers therefore

reflect the copy number representations of the target DNA sequence in the two genomic DNAs used for comparative hybridization.

Notably, using such an approach it is not required to
5 achieve sufficient signal from the different labeled genomic DNAs over each target sequence to allow meaningful fluorescence measurements. Consider that the test genomic DNAs on average yields five specific hybridization dots, while the reference genomic DNA yields one specific dot per target DNA sequence.
10 Then the conclusion seems valid that the copy number representation of the target sequence in question is five times higher in the test genomic DNA.

The ratio of dots from test and reference genomic DNA counted over a series of target DNA DNA fibers contained in a
15 given TNA-spot may deviate from the actual ratio of copy number representation of the target sequence in the test and reference genomic DNAs for a number of reasons. The differently labeled DNAs in the 1:1 hybridization mixture should be digested to the same size distribution. The number of background dots in the
20 vicinity of the target sequence should be approximately the same for both the test and reference genomic DNA. If necessary, the number of dots, which are expected to result from chance background dots on the target DNA sequence, should be calculated from the area surrounding the target DNA sequence
25 and subtracted from the overall number of counted dots. Only those background dots that are ordered exactly in line along a combed target DNA sequence can be confused with actual signal dots. The proposed approach to measure dots along combed DNA target DNA fibers thus helps strongly to minimize the number of
30 background dots, which could deteriorate the accuracy of counting the number of specific hybridization dots along a series of combed target DNA fibers. This is a decisive advantage of the proposed test as compared to a test where fluorescence ratios are determined from an entire DNA-spot
35 built up by a large number of non-combed DNA fibers.

Further, it should be emphasized that the proposed test allows the comparison of fluorescence or dot number ratios over a number of TNA-spots. TNA-spots, which contain combed Target DNA fibers present in equal copy number in both the test and reference genomic DNA, can serve to standardize the fluorescence ratios and dot ratios, respectively, not only with regard to the internal standardization in each individual TNA-spot given by the fluorescence or dot number obtained from the reference genomic DNA, but also with regard to the standardization of the data between different TNA-spots representing combed target DNA fibers present in equal and different copy numbers in the test and the reference genomic DNA.

In case that each target DNA fiber is covered with numerous specific dots, the target fibers can be easily distinguished as a linear array of dots. The unequivocal identification of the combed target DNA fibers is an absolutely essential requirement to count small numbers of dots, where a dot number ratio (or difference obtained by subtraction) is only meaningful when obtained from a series of target DNA fibers. Therefore the target fibers need to be visualized by other means when the number of dots is small, including specific DNA fluorochromes with an emission spectrum distinguishable from the fluorochromes used for the identification of hybridized DNA (or RNA) fragments.

Target DNA fibers can also be visualized by the admixture of labeled target DNA to the hybridization mixture. In this case a third label is required in addition to the two labels for the test and reference genomic DNA. The admixture of labeled target sequences has to be carefully adjusted in order to avoid too much suppression of the hybridization of the labeled target sequences present in the test and reference genomic DNAs. This problem can be avoided, if linkers are adapted to the target DNA sequence, which can be visualized by hybridization with a specific linker probe. The combed target

DNA sequence in question would then be embraced by two fluorescently labeled linker sequences. Alternatively, target DNA sequences can be cloned in the presence of hapten modified nucleotides, such as BrdU, and visualized immunocytochemically.

5 For the choice of useful target DNA sequences one should take into account that sequences that contain interspersed repetitive signals, e.g., Alu elements, require suppression hybridization, e.g., with an excess of unlabeled Cot1-DNA. It might be preferable to use target DNA fibers, which are
10 entirely specific for the genomic region in question, or to construct target DNA fibers devoid of interspersed repetitive elements.

CGH on combed DNA fibers bears the potential for an ultrahigh resolution CGH. Consider the following scenario: A
15 DNA fiber with known DNA sequence contains a target region of interest comprising a few hundred base pairs. We assume that the copy number of this target region is variable and may be higher (or lower) in the test genomic DNA as compared to the reference genomic DNA. We assume further that the positions of
20 the target and control regions along the DNA fiber are precisely mapped and that the DNA fiber is engineered in a way that its 5' - 3' orientation can be visualized, e.g., by probes to linker adapters of different size. In such a scenario the target region could be mapped by fractional length measurements
25 on each DNA fiber. Accordingly, one could identify dots which represent hybridization events to the target region and count the number of such events on a series of DNA fibers. This number should correlate with the representation of target sequences in the genomic test and reference DNA. In case that
30 the number target sequences is increased in the test genomic DNA as compared to the reference genomic DNA, the respective dot ratio should be increased over this region in contrast to other regions of the DNA fiber, for which we assume an equal copy number representation in both the test and reference
35 genomic DNA.

As a model system one could prepare PCR-amplified probes for two regions of a given DNA-fiber. DNA aliquots from each probe could be differently labeled and various hybridization mixtures prepared, where the differently labeled aliquots are present in different ratios. By counting the hybridization dots over the two target regions on a series of DNA fibers, one could determine to which extent the dot number ratios reflect the ratios of the differently labeled probe aliquots. This example may illustrate that an approach based on dot counting may be feasible to determine the copy number representation of very small target sequences where fluorescence ratios are no longer valid. This advantage is reemphasized by the following scenario.

Consider single-stranded DNA sequences containing a small (300 bp) target sequence of interest. Four CGH probe mixtures with differently labeled complementary 300 bp sequences are applied. On each individual fiber the target sequence of interest can only hybridized once, i.e., it will bear a dot of one color only. If the target is represented by double-stranded, denatured DNA, both strands serve as targets for a denatured double-stranded probe. Accordingly, the target region can hybridize with two 300 bp fragments at most. The resulting dots are either of the same color or of different color. While the measurement of a fluorescence ratio over the target region of a single fiber is obviously meaningless, the frequency with which the target region is covered by dots of the same (e.g., green or red) or of different colors (e.g., yellow) in a series of DNA fibers should be highly informative with regard to the frequency of differently labeled target sequences in the hybridization mixture.

Alternatively, DNA representing a target sequence of a few hundred base pairs could be fixed to the matrix the target spot and a fluorescence ratio could be determined from the entire spot. In this case, however, background could become a major problem. From these considerations we conclude that a dot

counting approach performed on a sufficient number of individual DNA fibers may be superior or even the only feasible way in case of ultrahigh resolution CGH.

In contrast to the largely variable size, form, and
5 relative position of individual target chromosomes considered in CGH on reference metaphase spreads, the length and orientation of a combed target DNA fibers can be strictly controlled. These defined patterns of combed target DNA fibers strongly facilitate their fully automated evaluation. In case
10 of a large number of TNA-spots, a colored print-out of fluorescence ratio or dot ratio measurements is recommended to facilitate the investigator's recognition of genomic regions, which are over- or under represented. In this print-out, each TNA-spot is represented by a colored spot. One color should
15 reflect TNA-spots with the range of fluorescence ratios apparently representing sequences present in balanced copy number in the test-DNA, a second color should reflect TNA-spots with sequences present in increased copy number, while a third color should reflect TNA-spots with sequences present in
20 decreased copy number. If desirable, color intensity may reflect the relative extent of over- or underrepresentation.

For a series of TNA-spots containing physically mapped sequences, the color spots could be arranged in a way that reflects mapping positions on chromosomes. For example, a
25 linear array of colored spots could represent the order of clones in a contig used for combed DNA fiber CGH. In case that the whole chromosome complement is represented by TNA-spots, the resulting color spots could be ordered as 24 linear arrays (representing chromosomes 1-22, X and Y). The color
30 spots within a given array then could represent the physical order of clones within the respective chromosome. The investigator then is enabled to see at one glance which chromosomes or chromosomal subregions are present in balanced, increased or decreased copy number.

**Potential Applications Of CGH On A
Matrix With Combed Target DNA Fibers**

In this section we will consider potential applications of CGH on combed target DNA fibers. In addition to potential studies of tumor DNA samples, we will also include applications in clinical genetics and cytogenetics.

Tests with gene specific TNA-spots would allow the rapid screening of test DNAs from tumor samples for copy number changes of specific genes. Given the appropriate equipment for the automated evaluation of TNA-spots, it may become feasible to evaluate DNA spot matrices with numerous spots representing the copy number representation of an entire set of genes of interest at a reasonable price.

A survey of whole genomes at the highest possible level of resolution would require such a high number of spots that such an approach appears impractical. For practical purposes, it may be advantageous to perform a survey of a test genomic DNA with unknown gains and losses by a series of tests with increasing resolution, starting by CGH on metaphase spreads and subsequently homing in on specific chromosome segments. In addition to CGH on reference chromosomes, matrices with spots representing composite DNA sequences of entire chromosomes, chromosome arms or bands may be applied. CGH performed directly on target DNA fibers provides the ultimate level of resolution for CGH and is only reasonable in cases where the screening of a very specific subset of target DNA sequences for copy number changes in a test genomic DNA is required. The following examples may illustrate how such a strategy could be applied.

CGH on reference chromosome spreads may reveal, for example, the non-random loss of a certain chromosome segment for a certain tumor entity. A consensus region can be defined by the comparison of all tumors showing this deletion. However, a considerable fraction of the tumors may not show any detectable deletion at this level of resolution (>10 Mbp). To

screen these tumors for much smaller deletions, a matrix with TNA-spots representing the consensus region could be used. The resolution would depend on the size and linear, genomic distance of the target sequences represented by a given matrix.

5 In many cases it should be sufficient for screening purposes to represent a chromosome region by a series of TNA-spots, where each spot defines for example a cosmid sequence a few hundred kb apart from the target DNA sequence contained in the next TNA-spot.

10 To achieve the highest, possible resolution even a whole contig can be represented by a series of TNA-spots. Fluorescence ratio measurements performed on a high resolution matrix representing a region of interest should help to define the cosmids, which represent the smallest deletion detectable

15 in this region of interest for a whole series of genic test DNAs obtained from patients with a specific tumor entity. This minimum deletion could be confirmed by FISH of the respective cosmids to tumor nuclei.

In this way, efforts of positional cloning of a suspected tumor suppressor gene could be strongly facilitated. For such

20 a purpose it would not be necessary to know the precise linear order of the cosmid clones representing the chromosome segment of interest. If, say, three TNA-spots with decreased fluorescence ratio would define the minimal detectable

25 deletion, one would expect that FISH with these three clones to extended chromatin fibers would confirm their vicinity. The latter approach could also be used to map the linear order of these cosmids.

Similarly, matrices with physically mapped cosmids

30 representing a chromosomal subregion could help to define amplified regions. Positional cloning of the genes involved in amplifications would be greatly facilitated, if the extension of such amplifications could be precisely mapped. Consider that a chromosome band has been identified as the source of the

35 amplified sequences by CGH to reference metaphase spreads.

Applying a matrix with a series of physically mapped clones representing the chromosome band in question should yield increased fluorescence ratios for any TNA-spot representing DNA sequences, which are in fact amplified. Normal fluorescence ratios should be measured for all other TNA-spots. The extent of the amplification could then be defined by the two TNA-spots with increased fluorescence ratios, which represent the most proximally and most distally mapping clones.

In the future, matrices containing TNA-spots with combed target DNA fibers for the copy number representation of oncogenes and tumor suppressor genes can be developed. The choice of the target sequences for a given matrix will depend on the tumor entity and the demands of the test. For example, matrices can be specifically developed to identify gains or losses with prognostic value (e.g., N-myc amplifications or 1p36- deletions in neuroblastomas). For some tumors, e.g., colorectal tumors, knowledge about the relevant genes involved in tumor initiation and progression seems already sufficiently advanced to consider the development of such a strategy. For other tumors we still lack such knowledge. CGH on combed DNA fibers may help to obtain such knowledge in the future and to perform large scale tests performed with the aim to correlate the patterns of relative copy number changes of genomic DNA sequences (and changes in the number of specific mRNAs) in tumor cells with the clinical course of the disease. Ideally, matrices should be developed, which contain TNA-spots representing all genes that are relevant for the biological properties of the tumor entity in question.

High resolution matrices could also open new avenues in clinical cytogenetics. Two examples may be sufficient to demonstrate the range of possible applications. A CGH test-matrix could be developed to screen DNA from patients with phenotypes suspicious for unbalanced chromosome aberrations. Taking into account that unbalanced rearrangements often include terminal chromosome segments, a CGH-matrix containing

TNA-spots with combed target DNA fibers representing cloned sequences from each individual chromosome end may become a great clinical value. As another example, consider the case of a carrier-analysis for X-linked recessive diseases. A boy, who suffers from Duchenne muscular dystrophy, may be the first victim of that disease in a family. In some 60% of the cases a deletion can be found as the cause of the mutation. The question has then to be answered whether his disease is due to a germ cell mutation or whether his mother is already a carrier. The consequences for genetic counseling are totally different and in the latter case other female members, e.g., the sisters of the boy's mother, may also be concerned about their carrier-status. A matrix with a series of cosmids spanning the entire dystrophin gene could potentially provide a reliable and automated procedure for carrier screening in deletion prone cases of DMD.

**Comparative RNA Hybridization On Combed
DNA Fibers: A New Method For The Assessment
Of Expression Levels Of Tumor Relevant Genes**

While CGH on combed DNA fibers should provide information on deleted or amplified genes, it would not detect the silencing or overexpression of genes in tumor cells as compared to their normal counterparts. We propose an approach to study the expression status of genes by comparative RNA hybridization on combed DNA fibers. Consider for example a scenario where an amplification is detected in a given tumor entity. Amplicons may be large and contain several genes. It may not be clear which gene(s) of these genes are strongly expressed. A DNA-spot matrix containing combed cDNA-fibers for the coding sequences of all genes in question can be used for comparative nucleic acid hybridization with differently labeled RNA-preparations (or corresponding cDNA preparations) from the tumor and a normal reference tissue. The resulting fluorescence or dot number ratios then can provide insight in the (relative) expression status of the tested genes in the

tumor as compared to normal tissue. The same approach could be used to detect point mutations which interfere with the description of a gene.

This invention will now be described in greater detail in the following Examples.

EXAMPLE 1

Preparation Of Glass Slides With Combed DNA-fibers

Any cloned DNA, PCR-amplified DNA or other purified DNA can be used for DNA-combing depending on the purpose of the comparative hybridization experiment. The amount of DNA necessary per TNA-spot is small, since only a small number of DNA-fibers is needed for evaluation is combed fiber CGH experiments (for example, 1 ng of cosmid DNA (40kb) contains 2.4×10^2 molecules). In the following we describe a typical experiment with cosmid DNA.

Attachment of target DNA fibers requires glass surfaces pretreated by salinization as described previously (Bensimon et al. 1994). Prior to their attachment, cosmids can be stained for 1 hour at room temperature with YOYO-1 (Molecular probes; Cat. No. Y-3601). Staining solution was freshly prepared as follows: 1[^]ml DNA (1mg/ml) + 33[^]ml YOYO-1 diluted 1:100 in $T_4O_4E^-$, + 66[^]ml $T_4O_4E^-$; $t_4O_4E^-$ buffer contains 40 mM Tris-Acetate, 2 mM EDTA, pH 8.0). YoYo-1 stained cosmid DNA was diluted in 50 mM MES-buffer, pH 5.5, and then attached to the glass surface (note that the pH is a most critical point for successful attachment).

In our present experiments, each glass slide (22 x 22 mm) contained only one type of combed DNA fibers. Where appropriate, several glass slides containing DNA fibers with different sequences were processed in parallel.

For the preparation of a single matrix with a series of TNA spots with combed DNA fibers, salinization of the glass surface can be restricted to the areas selected for the positions of the TNA spots. A series of droplets, each containing DNA fibers with the required DNA target sequence for

a given area, can be put on these preselected areas. Target fibers contained in each droplet are allowed to attach to the surface. The excess fluid with non-attached fibers is removed taking care that the surface is kept wet.

5 Following DNA fiber attachment, combing was carried out as described (Bensimon et al. 1994). Microscopic visualization of YoYo-1 stained fibers allowed a control of the density and direction of attached fibers. Slides with combed DNA fibers were baked at 60° C for at least 4 hours and treated with a
10 "blocking solution" consisting of 3% BSA in 2xSSC at 37°C for 30 min. After a brief wash with 2xSSC, slides were put through a series of 70%, 90%, 100% E:OH, 2 min each and air dried. Storage of the dried slides is recommended in sealed boxes at +4°C.

15 EXAMPLE 2

Comparative Hybridization To Combed DNA-fibers

Comparative genomic hybridization (CGH) to combed DNA fibers was essentially carried out as described elsewhere for CGH to metaphase chromosomes (du Manoir et al. 1993, 1995). Briefly, test and reference genomic DNAs were nicktranslated with biotin and digoxigenin, respectively. Alternatively, the DNAs can be labeled directly with appropriate fluorochrome conjugated nucleotides. Combed DNA fibers were denatured for 2 min. at 72°C in 70% FA/0.6xSSC, pH7.0. Thereafter, slides were put through a series of ice cold EtOH (70%, 90%, 100%) and air dried. Ten μ l hybridization mixture (containing 500 ng each of the test and reference genomic DNA, 50 μ g of Cot1 fraction of human DNA (BRL/Life Technologies) and 55 μ g sonicated salmon testes DNA (Sigma) in 50% formamide, 1xSSC and 10% dextrane sulphate) were put on each glass slide (22 x 22 mm) with combed DNA fibers. Another slightly smaller glass slide (18 x 18 mm) was put on top and sealed with fixogum.

Hybridization was carried out overnight at 37°C. Washing and detection procedures for biotin and digoxigenin labeled sequences were carried out as described (Lichter and Cremer

1992, du Manoir et al. 1993, 1995) with minor modifications. Slides were washed 3 x 5 min. with 50% FA/SSC and another 3 x 5 min. with 2SSC at room temperature. Following equilibration in 4xSSC/0.1% Tween 20 at 37°C slides were incubated for 30 min. with 3% BSA/4SSC/0.1% Tween 20 at 37°C (a blocking step to reduce background). Slides were then washed for 5 min. in 4xSSC/0.1% Tween 20 at 37°C and incubated with avidin DCS conjugated to FITC (Vector Laboratories) for 45 min. at 37°C to visualize biotin labeled probes.

Digoxigenin-labeled probes were detected by incubation with mouse-anti-digoxin IgG antibodies as the primary antibody (Sigma), followed by incubation with a sheep-anti-mouse IgG antibody conjugated with the fluorochrome CY3. In between these steps slides were washed 5 min. each in 4xSSC/0.1% Tween 20 at 37°C. If necessary, avidine-FITC signals were amplified as described (Pinkel et al. 1986). Finally, slides were air-dried and mounted in Vectashield (Vector Laboratories) as an antifade.

In a series of model experiments, probe mixtures consisting of different ratios of biotin labeled and digoxigenin labeled cosmid sequences (e.g., 1:1 (20 ng + 20 ng), 2:1 (20 ng + 10 ng), 5:1 (20 ng + 4 ng) and 10:1 (20 ng + 2 ng) were used for in situ hybridization to combed DNA fibers representing the same cosmid as target sequence.

EXAMPLE 3

Evaluation Of Combed DNA Fibers

Subjected To Comparative Hybridization

(A) Image acquisition

Grey-level images were recorded with a cooled black and white CCD camera (Photometrics) separately for each fluorochrome. Optimal exposure times and optical settings were established empirically and then kept constant for the entire set of DNA fibers recorded for a given experiment. Images were stored under FITS-format.

(B) Image processing

(1) Measurement of fluorescence ratios

Digital images were processed by NIH image (version 1.59b9). A rectangular mask was adapted to each DNA fiber. Within the mask two integrated fluorescence values were obtained as the sum of the grey level values for all pixels. Background fluorescence intensity was determined after shifting the mask to the immediate neighborhood of a given DNA fiber. Fluorescence intensities were corrected by background subtraction. The fluorescence ratio for each fiber was calculated by dividing the corrected FITC fluorescence intensity with the corrected Cy3 fluorescence intensity. Fifty DNA fibers were evaluated in a typical experiment to calculate the mean fluorescence ratio value.

(2) Dot counting

Notably, the labeling observed along combed DNA fibers in the experiments described above is not homogeneous. Instead signal dots probably representing hybridized labeled DNA fragments can be distinguished on these fibers. For dot counting, digital images were thresholded and gravity centers were determined.

In summary, this invention provides procedure termed combed DNA fiber comparative hybridization (CFCH). For this new procedure, there is provided

- 1) A possibility to measure the relative copy number representation of DNA sequences from labeled genomic test DNA (e.g. tumor DNA, DNA from patients with imbalanced types of chromosome aberrations) as compared to the copy number representation in differently labeled normal genomic DNA with a resolution in the kilobase pair range, i.e. at the level of single genes. In contrast, CGH as described before can

detect copy number changes only in the megabase pair range.

- 2) A possibility to measure copy number differences in mRNAs transcribed from specific genes by comparative hybridization of a mixture of mRNAs prepared from test and reference cells.
- 3) The arrangement of target DNA spots with combed DNA fibers representing genomic or cDNA test sequences of interest on a suitable matrix for the simultaneous testing of multiple sequences for their relative copy number representation in the hybridization mixture in a geometrical format, which makes such an arrangement particularly useful for automated evaluation.

The invention is particularly useful for the detection of genetic diseases in eucaryotic cells.

REFERENCES

- Bensimon, A., Simon, A., Chiffaudel, A., Croquette, V., Heslot, F., Bensimon, D. Science 265:2096-2098 (1994).
- du Manoir, S., Speicher, M.R., Joos, S., Schrock, E., Popp, S., Dohner, H., Kovacs, G., Robert-Nicoud, M., Lichter, P., Cremer, T. Hum. Genet. 90:590-610, (1993).
- du Manoir, S., Schrock, E., Bentz, M., Speicher, M.R., Joos, S., Ried, T., Lichter, P. Cremer, T. Cytometry 19:27-41 (1995).
- Joos, S., Scherthan, H., Speicher, M.R., Schlegel, J., Cremer, T., Lichter, P. Hum. Genet 90:584-589 (1993).
- Isola, J., DeVries, S. Chu, L., Ghazvini, S., Waldman, F. Am. J. Path. 145:1301-1308 (1994)
- Kallioniemi, A., Kallioniemi, O.P., Sudar, D., Rutovitz, D., Gray, J.W., Waldman, F., Pinkel, D. Science 258:818-821 (1992).
- Kallioniemi, O.P., Kallioniemi, A., Piper, J., Isola, J., Waldman, F.M., Gray J.W., Pinkel, D. Genes, Chromosomes and Cancer 10:231-243 (1994).
- Kallioniemi, O.P., Kallioniemi, A., Sudar, D., Rutovitz, D., Gray, J.W., Waldman, F., Pinkel, D. Sem. Cancer Biol. 4:41-46 (1993).
- Kallioniemi, A., Kallioniemi, O.P., Piper, J., Tanner, M., Stokke, T., Chen. L., Smith, H.S., Sudar, D., Pinkel, D., Gray, J.W., Waldman, F. Proc. Natl. Acad. Sci. USA 91:2156-2160 (1994).
- Lichter, P., Cremer, T., Borden, J., Manuelidis, L., Ward, D.C. Hum. Genet. 80:224-234 (1988).
- Lichter, P., Cremer, T.: Chromosome analysis by non-isotopic in situ hybridization. In: Human cytogenetics: A practical approach; eds.: Rooney, D.E., Czepulkowski, B.H., IRL Press, Oxford:157-192 (1992).
- Muleris, M. Almeida, A., Dutrillaux, A.M., Pruchon, E., Vega, F., Delattre, J.Y., Poisson, M. Malfoy, B., Dutrillaux, B. Genes, Oncogene 9:2717-2722 (1994).

Pinkel, D., Landegent, J.; Collins, C. Fuscoe, J.,
Segraves, R. Lucas, J., Gray, J. Proc. Natl. Acad. Sci. U.S.A.
85:9138-9142 (1988).

Piper, J., Rutovitz, D., Sudar, D., Kallioniemi, A.,
5 Kallioniemi, O.P., Waldman, F.M., Gray, J.W. Pinkel, D.
Cytometry 19:10-26 (1995).

Ried, T., Petersen, L., Holtgreve-Grez, H., Speicher,
M.R., Schrock, E., du Manoir, S., Cremer, T. Cancer Res.
54:1801-1806 (1994).

10 Schrock, E., Thiel, G., Lozanova, T., du Manoir, S.,
Meffert, M.C., Jauch, A., Speicher, M.R. Nurnberg, P. Vogel,
S., Janisch, W., Donis-Keller, H., Ried, T., Witkowski, R.,
Cremer, T. Am J. Path. 144:1203-1218 (1994).

15 Speicher, M.R., du Manoir, S., Schrock, E., Holtgreve-
Grez, H., Schoell, B., Lengauer, C., Cremer, T., Ried, T. Hum.
Mol. Genet. 2:1907-1914 (1993).

Speicher, M.R., Prescher, G., du Manoir, S., Jauch, A.,
Horsthemke, B., Bornfeld, N., Becher, R., Cremer, T. Cancer
Res. 54:3817-3823 (1994).

20 Speicher, M.R., Jauch, A., Walt, H., du Manoir, S. Ried,
T., Jochum, W., Sulser, T. Cremer, T. Am J. Path. 146: 1332-
1340 (1995).

What is claimed is :

1. A method for detecting and quantifying the presence of a nucleic acids sequence in a genome comprising:

5 (A) immobilizing by combing the target nucleic acids sequence on a supportive matrix,

(B) contacting the combed nucleic acids sequence with a 1:1 mixture of differently labeled nucleic acids probe to be tested and reference nucleic acids probe to form hybridization,

10 (C) counting individual labeled hybridization dots located on combed nucleic acids sequence,

(D) determining the number of the nucleic acids sequence to be detected and quantified by the calculation of the ratio between the total dot number from the test probe and the total dot number from the reference probe.

15 2. A method according to claim 1, wherein the combing of the target sequence comprises:

(A) anchoring the end of said sequence to a supportive matrix,

20 (B) contacting the anchored sequence with a liquid to form a meniscus,

(D) combing said sequence by a receding air-water interface.

3. A method according to claim 2, wherein said liquid has a pH about 5 to about 10 to form a meniscus.

25 4. A method according to claims 1 to 3, wherein the target sequence is labeled.

5. A method according to claims 1 to 4, wherein the combed target sequence is DNA and test and reference probe are RNA- cDNA- or genomic DNA-preparations.

30 6. A method according to anyone of claims 1 to 5, wherein the nucleic acids sequence to be detected and quantified is a human oncogene.

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| Int. Patent Application No PCT/IB 96/01219 | | |
| A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12Q1/68 | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12Q | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| P,X | WO 96 17958 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 13 June 1996 see the whole document --- | 1-6 |
| X | WO 93 18186 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 16 September 1993 see the whole document --- | 1,4-6 |
| X | WO 95 21939 A (INSTITUT PASTEUR) 17 August 1995 see the whole document, especially page 14 line 12 - page 17, line 6 and pages 19-21 --- -/-- | 1-3 |
| <div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div> | | |
| * Special categories of cited documents : <div style="display: flex;"> <div style="flex: 1;"> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*I* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>*Z* document member of the same patent family</p> </div> </div> | | |
| Date of the actual completion of the international search | Date of mailing of the international search report | |
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| Name and mailing address of the ISA | Authorized officer | |
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016 | De Kok, A | |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|---|-----------------------|
| X | CELLULAR AND MOLECULAR BIOLOGY, vol. 41, no. 7, November 1995, PARIS FR, pages 925-931, XP000197050 C. DESMAZE ET AL.: "In situ hybridization of fluorescent probes on chromosomes, nuclei or stretched DNA: applications in physical mapping and characterization of genomic rearrangements" see the whole document --- | 1-6 |
| X | SCIENCE, vol. 270, no. 5235, 20 October 1995, LANCASTER, PA US, pages 467-470, XP000644675 M. SCHENA ET AL.: "Quantitative monitoring of gene expression patterns with a complementary DNA microarray" see the whole document --- | 1-6 |
| A | WO 95 09929 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 13 April 1995 see the whole document ----- | 1 |

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|--|--|
| WO 9617958 A | 13-06-96 | NONE | |
| WO 9318186 A | 16-09-93 | CA 2131543 A EP 0631635 A JP 7505053 T | 16-09-93 04-01-95 08-06-95 |
| WO 9521939 A | 17-08-95 | FR 2716206 A FR 2716263 A AU 1712095 A AU 1814595 A EP 0744028 A EP 0743988 A WO 9522056 A | 18-08-95 18-08-95 29-08-95 29-08-95 27-11-96 27-11-96 17-08-95 |
| WO 9509929 A | 13-04-95 | US 5472842 A CA 2171110 A EP 0722507 A | 05-12-95 13-04-95 24-07-96 |

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